

## INACTIVATION IN *MYXICOLA* GIANT AXONS RESPONSIBLE FOR SLOW AND ACCUMULATIVE ADAPTATION PHENOMENA

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### SUMMARY

1. The action potential in *Myxicola* giant axons is abolished if the nerve is stimulated at frequencies higher than about  $5 \text{ sec}^{-1}$ . At  $1 \text{ sec}^{-1}$  the magnitude of the action potential is not maintained upon sequential stimulation but decreases until the response is abolished.

2. The behaviour of the ionic currents underlying the action potential was studied with voltage-clamp techniques to find the origin of such adaptation. These studies showed a frequency-dependent decline of the sodium currents.

3. The decline in the Na currents upon repetitive depolarization is shown to be due to a decrease in the Na conductance and not to change in driving force.

4. An analysis of the effects of conditioning depolarizations on the Na current during a depolarizing test pulse demonstrates that in a single short depolarization (less than 10 msec) 15 % of the Na conductance enters an inactivated state from which recovery is very slow. Upon repetitive depolarizations the amount of Na conductance available accumulates in this slowly recovering inactivated state.

5. The data are explained by proposing that every time the membrane is depolarized open channels undergo one of two competing reactions. Open channels enter either the traditional inactivated state described by Hodgkin & Huxley (1952*b*) from which recovery is fast (a few milliseconds) or an inactivated state from which recovery is very slow (seconds). In *Myxicola*, only 15 % of open channels enter the later inactivated state in a single depolarization. Upon repetitive depolarizations, however, the fraction in this state accumulates if the frequency of pulsing is faster than the rate of recovery.

6. Axons in which the amount of open channels entering the slowly recovering inactivated state is significant, such as in *Myxicola*, have thus a system capable of storing the previous activity of the axon for periods of seconds or minutes.

### INTRODUCTION

'*Myxicola* when first picked up will twitch violently, but after several twitches the worm will lie motionless in the hand' (Dales, 1970). Adaptation phenomena of various types are well documented in Annelids (Dales, 1970; Horridge, 1959). If

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*Myxicola* giant axons are stimulated at room temperature, at frequencies higher than  $1 \text{ sec}^{-1}$  the magnitude of the action potential decreases. After a few spikes, the response is abolished but the threshold fully recovers if the axon is left at rest for several seconds. A new train of stimuli will elicit the same response.

Potassium accumulation in an extracellular space could produce a decay in the magnitude of the action potential during a train of stimuli as it does in squid axons at much higher frequencies (Frankenhaeuser & Hodgkin, 1956). The K accumulated in the extracellular space depolarizes the membrane and increases the inactivation of the Na conductance. However, the action potentials in *Myxicola* decrease in magnitude without any apparent change in the resting potential or in the negative afterpotential.

Under voltage clamp the Na currents in *Myxicola* behave differently from those in squid or in the node of Ranvier in frog nerve. The magnitude of the Na current decreases reversibly during trains of depolarizing pulses at frequencies of the order of  $1 \text{ sec}^{-1}$ . Numerical calculations demonstrate that this effect can account for the change in threshold during a train of action potentials.

*Myxicola* giant axons achieve such a behaviour by a modification in the kinetics of the voltage-dependent Na conductance (Hodgkin & Huxley, 1952*b*). The characteristics of this modification are described in this paper. A preliminary report has been published (Rudy, 1975).

#### METHODS

##### Material

The experiments were performed with *Myxicola infundibulum* of both sexes collected on the coast of the English Channel near Plymouth. The specimens were stored for up to 4 months in circulating sea water at  $8^\circ\text{C}$ . Except for a seasonal variation in the resting potential (more negative during the winter, over a period of two years) no differences were observed which could be related to sex, size or time under captivity.

The mean values of resting potential of axons used was  $-72 \text{ mV}$  (range  $-58$  to  $-81 \text{ mV}$ ). Mean diameter was  $760 \mu\text{m}$  (range  $500$ – $1100 \mu\text{m}$ ). The size of the action potential ranged between  $100$  and  $120 \text{ mV}$ .

The giant axon was dissected by a variation of the method described by Binstock & Goldman (1969). For most experiments the worm was anaesthetized by immersion in 5% ethanol in sea water at  $5^\circ\text{C}$  for 30 min. After the nerve cord had been separated from the gut (see Binstock & Goldman, 1969) collagenase was applied. A solution containing collagenase type III (Sigma Chemicals;  $50$ – $100 \text{ u./ml.}$  in K-free artificial sea water) was applied to the dorsal surface of the nerve cord for 15 min. At the end of the treatment the specimen was washed with sea water. With this treatment the connective tissue which surrounds the axon on its dorsal surface and which contains the dorsal blood vessel became loose. So did the tightly attached bands of connective tissue which constrict the axon at each intersegment. This procedure made the dissection of the constrictions and the dorsal blood vessel very easy. These could be stripped off by simply pulling with fine forceps. Large pieces of nerve could be cleaned, after which the procedure of Binstock & Goldman (1969) was followed to remove the nerve cord from the animal. Control experiments demonstrated that the collagenase treatment did not affect the survival of the axon, the size and shape of the Na and K currents, the characteristics of the inactivation of the Na conductance or the leak currents. In these cases, the axons were dissected without enzymatic treatment and the behaviour tested before and after the application of collagenase to the solution bathing the axon in the experimental chamber. The axon was cleaned of surrounding muscle fibres as described by Binstock & Goldman (1969).

##### Experimental procedure

*Myxicola* axons were studied under voltage clamp with techniques similar to those used for squid axons (Rudy, 1978). The values of series resistance ranged between  $12$  and  $18 \Omega\text{cm}^2$ . Series resistance

compensation was used in most of the experiments. The compensation was increased to near the point of ringing of the potential trace. Compensation could be obtained for 50–72 % of the series resistance.

For quantitative studies, the values of Na or Na + K currents were corrected by subtracting the leakage current. This was obtained by repeating the experiment in the presence of tetrodotoxin (TTX) or from values obtained from hyperpolarizing pulses assuming linearity for the leakage current.

#### *Nomenclature*

Potentials are referred to the external solution as ground, so that the resting potential is negative. Potential changes in the positive direction are referred to as depolarizations, negative ones as hyperpolarizations. 'Potential below' means more negative than.... Outward currents are shown as upward deflexions; inward currents go downwards.

#### *Solutions*

Solutions were prepared with distilled water and stored at 4 °C. Artificial sea water (ASW) contained 430 mM-NaCl, 10 mM-KCl, 10 mM-CaCl<sub>2</sub>, 50 mM-Mg Cl<sub>2</sub> and 5 mM. Tris-HCl. K-free ASW contained 10 mM additional NaCl instead of KCl. 4-aminopyridine (4-AP) solutions were stored in dark bottles and used within two weeks.

### RESULTS

As in the squid giant axon (Hodgkin & Huxley, 1952*b*) under voltage-clamp conditions, four components of current are obtained in *Myxicola* giant axons: a capacitance transient at the beginning and end of the pulse, and three components of ionic current; an early inactivating current carried mainly by Na<sup>+</sup> ions, a late current which is maintained for the duration of the pulse and carried mainly by K<sup>+</sup> ions, and a time-independent leak. The kinetics of the various current components are very similar to those in squid axon (Goldman & Schauf, 1973).

Fig. 1 illustrates the behaviour of the ionic currents in *Myxicola* axons when repetitive depolarizations are applied. The Na currents decrease with subsequent depolarizations to a final value. In contrast the K currents do not change during the train. The amount of the decrease and the rate of decrease of the Na current increase as the frequency of the depolarizations increases. The original value of the Na current is recovered if the holding potential is restored for 15–30 sec. The phenomenon can be reproduced quantitatively many times in the same axon.

In some records the Na current appears to rise faster as its magnitude decreases upon repetitive depolarization. This is very probably an artifact due to uncompensated series resistance. It is less conspicuous in axons with better series-resistance compensation. An experiment to test this point further was performed in two axons. After recording the Na currents during a train of depolarizations, a small amount of TTX was added to block 70–80 % of the Na current and thus decrease effects of series resistance. The Na currents were then recorded at fast sweep during a train of depolarizing pulses and were found to decrease without any change in their time course.

#### *Origin of the response of the sodium currents to repeated stimulation*

Our present knowledge of the mechanisms controlling membrane currents allows several possibilities to explain the result illustrated in Fig. 1. Some of these are specific processes not directly related to the membrane structures responsible for the conductance changes. Thus it seems appropriate in the first instance to consider these various possibilities.

A slowly activating outward current could produce an apparent decrease in the magnitude of the Na currents with subsequent depolarizations. Although this is

unlikely, since the time course of the Na currents does not change and since there is no change on the K currents, two experiments were performed to test such a possibility. In an axon poisoned with TTX to block the Na currents, repetitive pulsing

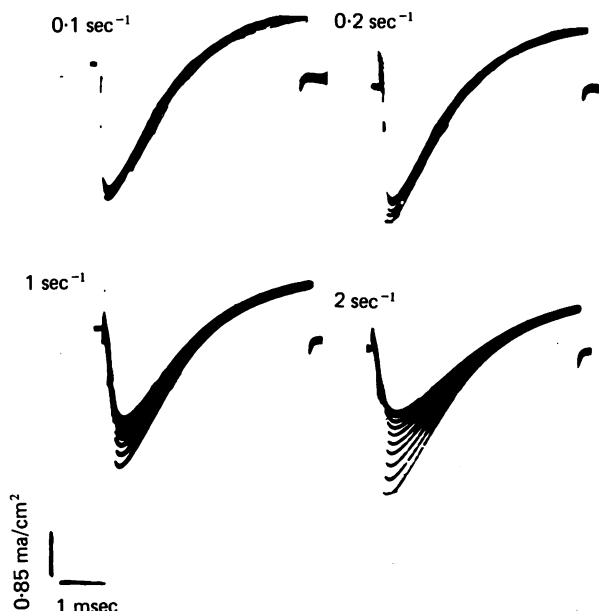


Fig. 1. Frequency response of ionic currents in *Myxicola* giant axons. Axon bathed in K-free ASW.  $V_H = -75$  mV.  $V_M$  during the pulse  $= -20$  mV. Temp.  $8.1^\circ\text{C}$ . The frequency of pulse application is indicated next to each set of records.

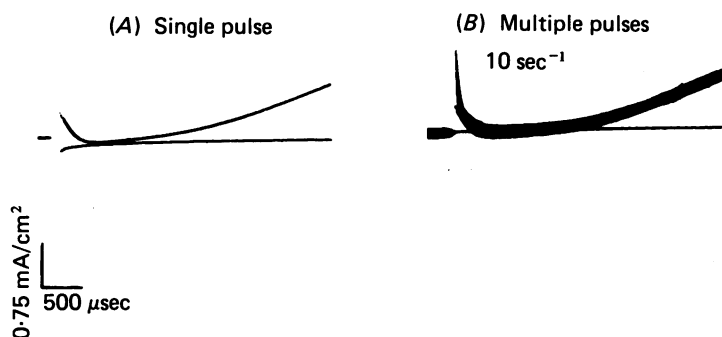


Fig. 2. Repetitive depolarization in a TTX-poisoned axon. Expt. 15-N-75. Axon bathed in K-free ASW with  $3\ \mu\text{M}$ -TTX.  $V_H = -80$  mV,  $V_M = 20$  mV. *A*, currents for single depolarizing and hyperpolarizing pulse of 100 mV. *B*, currents recorded during a train of depolarizing pulses of 100 mV at frequency of  $10\ \text{sec}^{-1}$  for 3 sec. Temp.  $7^\circ\text{C}$ .

does not show any increasing current (Fig. 2). The frequency effect 'reverses' at the Na equilibrium potential; there is no other ion with such a reversal potential (Rudy, 1975; and see Fig. 3).

Potassium accumulation produced by the late outflow of K during a depolarizing

pulse could produce inactivation by other means different from a change in the resting potential. Adelman & Palti (1969) have shown that external K might produce a pharmacological effect on the Na currents, which is removed by hyperpolarization. However, the decrease in the magnitude of the Na currents with subsequent depolarizations illustrated in Fig. 1 is identical whether the K currents are present

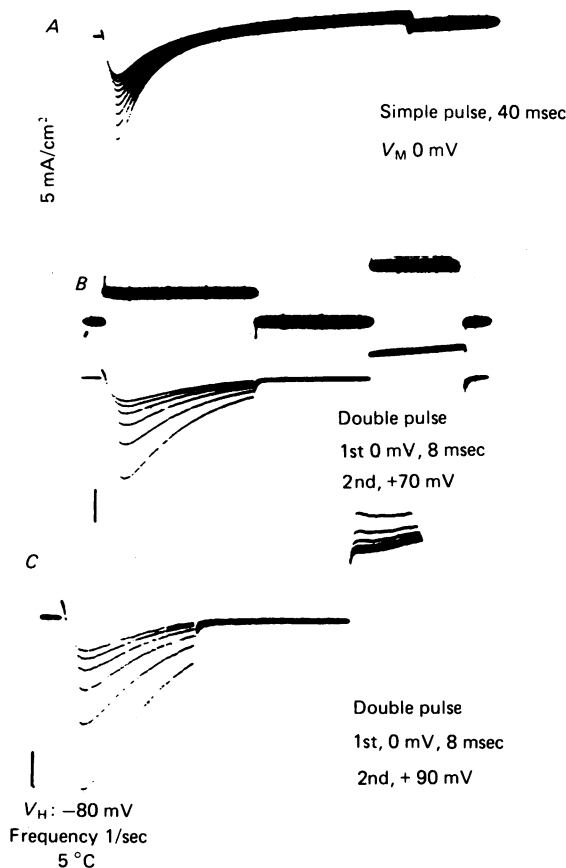


Fig. 3. Comparison of the frequency response of the Na currents for double pulses and for a long single pulse. Axon bathed in K-free ASW and  $10 \text{ mM}$ -4-AP.  $V_H = -80 \text{ mV}$ . Potential during the pulse and duration of the pulse are indicated next to each set of records. Note: the difference in the rate of decay of the Na currents for the single long pulse (A) with that for double pulses. In B the second pulse took the membrane potential to the Na equilibrium potential.

or not. Potassium currents were blocked by external application of 4-AP (Pelhate & Pichon, 1974). Quantitative estimations of the frequency response of the Na currents before and after the application of 4-AP showed no difference (Table 1).

Calculations of the size of the extracellular space of the axons used here by a method similar to one used by Frankenhaeuser & Hodgkin (1956) lead to a mean value of  $2821 \text{ \AA}$ , i.e. about ten times larger than the Frankenhaeuser-Hodgkin space (mean  $290 \text{ \AA}$ ) of squid axon. This makes accumulation an unlikely hypothesis.

Finally, as illustrated by Fig. 3 accumulation of Na in an intracellular space (or depletion from an extracellular space) cannot account for the frequency response of the Na currents. The Na current decreases without any change in the Na reversal potential. In this experiment two pulses, separated by an interval of a few milliseconds (to allow recovery from fast inactivation, 'h' inactivation, Hodgkin &

TABLE 1. Frequency response of the sodium currents before and after blockage of potassium currents with 4-AP

Expt.	Frequency (sec <sup>-1</sup> )	Before addition of 4-AP		After addition of 4-AP	
		$I_{pf}/I_{po}$	$\tau$ (sec)	$I_{pf}/I_{po}$	$\tau$ (sec)
23-N-74	0.25	0.87	—	0.84	—
	0.5	0.80	—	0.81	—
	0.1	0.73	3.4	0.70	3.9
	0.2	0.58	2.63	0.60	2.86
	0.4	0.41	1.75	0.39	1.79
14-N-75	1	0.65	3.8	0.60	3.9
	5	0.40	1.4	0.42	1.6
	10	0.28	0.89	0.31	0.96

$I_{pf}$  = peak Na current for the steady-current after a train.

$I_{po}$  = peak Na current for the first pulse in the train.

$\tau$  = time constant of decay of peak Na current from  $I_{po}$  to  $I_{pf}$ .

Huxley, 1952*a, b*) were applied to the membrane. The first pulse took the membrane potential below the Na reversal potential, the second to the Na reversal potential (*B*), or to a potential above it (*C*). The two pulse sequence was repeated at 1 sec<sup>-1</sup>. The current during the second pulse should grow (in the outward direction) if the decrease during the first pulse was produced by accumulation of Na inside (or depletion outside). No change is observed in *B*, and a decrease rather than an increase in *C*. A simple calculation shows that the expected change would be quite considerable. For the case shown in *B* an outward current equal to a third of the current in the first pulse should be obtained for the second pulse during the second depolarizing series. Since the membrane potential is the same for any of the pulses in a train and, as shown above, there is no change in the reversal potential, the decrease of the Na currents with repetitive stimulation must be produced by a decrease in the membrane conductance for Na<sup>+</sup> ions.

It is clear from the experiment in Fig. 1 that the decrease in conductance is produced by a process which occurs during the short time that the membrane is depolarized in each pulse and recovers when the membrane is hyperpolarized. The longer the interval between depolarizing pulses (the smaller the frequency of pulsing) the smaller the effect because there is more time for the process to recover, etc. In the terminology of Hodgkin & Huxley (1952*a, b*), this type of reduction in the conductance available for a depolarizing pulse induced by a previous depolarizing pulse is produced by inactivation processes controlling the membrane conductance. In contrast to the normal Na inactivation (*h*), however, this inactivation in *Myxicola* recovers very slowly but it seems to be induced very fast (by relatively short

depolarizing pulses). The characteristics of the onset and recovery of this inactivation (*s*) will be described in more detail below.

*Influence of the membrane potential on the decrease of the Na conductance upon repetitive stimulation*

As illustrated in Fig. 4, the decrease of the Na currents with repetitive stimulation is dependent on the membrane potential during the interval between the depolarizing pulses (holding potential  $V_H$ ). In contrast, changing the potential during the pulse,

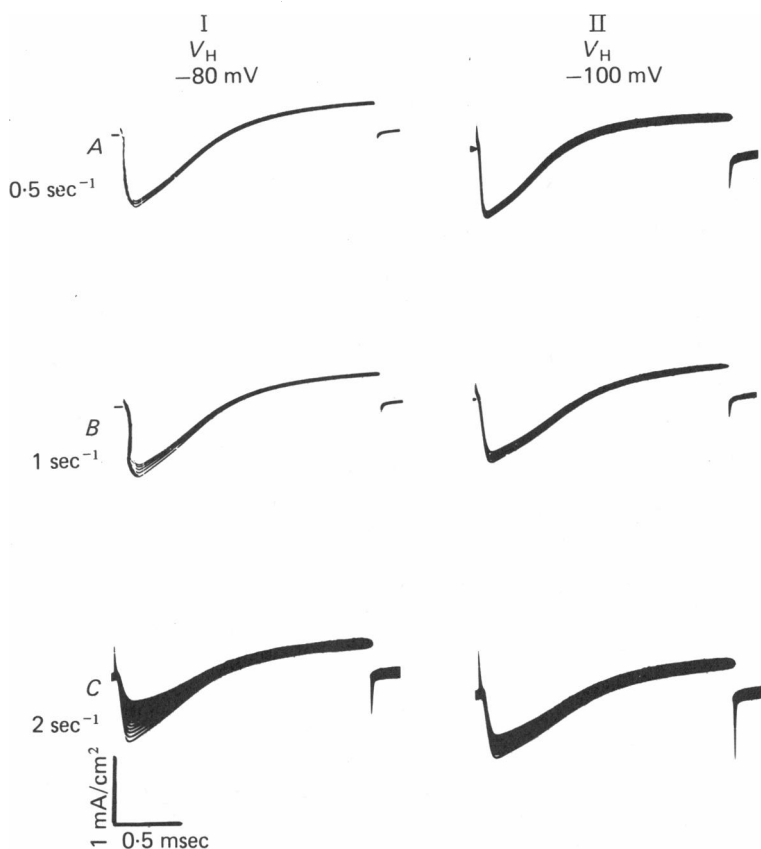


Fig. 4. Effect of holding potential ( $V_H$ ) on the frequency response of the Na currents. Expt. 11-A-75. Axon bathed in K-free ASW. Temp. 7.5 °C. Pulses to +20 mV were applied from a  $V_H$  of -80 mV (I) or -100 mV (II) at the frequencies indicated. Calibrations: 1 mA/cm<sup>2</sup> and 0.5 msec.

but maintaining a constant holding potential, did not seem to influence significantly the frequency response (Fig. 5). Thus it seems that the effect that is induced during a depolarizing pulse saturates for potentials below the lower tested or does not depend on potential. On the other hand the recovery is faster if the potential is more negative. This behaviour is similar to that of the fast inactivation (*h*). Both in squid and in *Myxicola* the steady value of *h* and the time constant of *h* saturates around -30 to

$-20$  mV and the recovery is faster if the membrane potential becomes more negative (Hodgkin & Huxley, 1952*b*; Goldman & Schaaf, 1973).

It is also necessary to verify that the rate of onset of the phenomenon is fast, as proposed above. One of the observations is illustrated in Fig. 6. In this experiment a depolarizing pulse to  $+20$  mV was repeated at a frequency of  $1 \text{ sec}^{-1}$ . This was

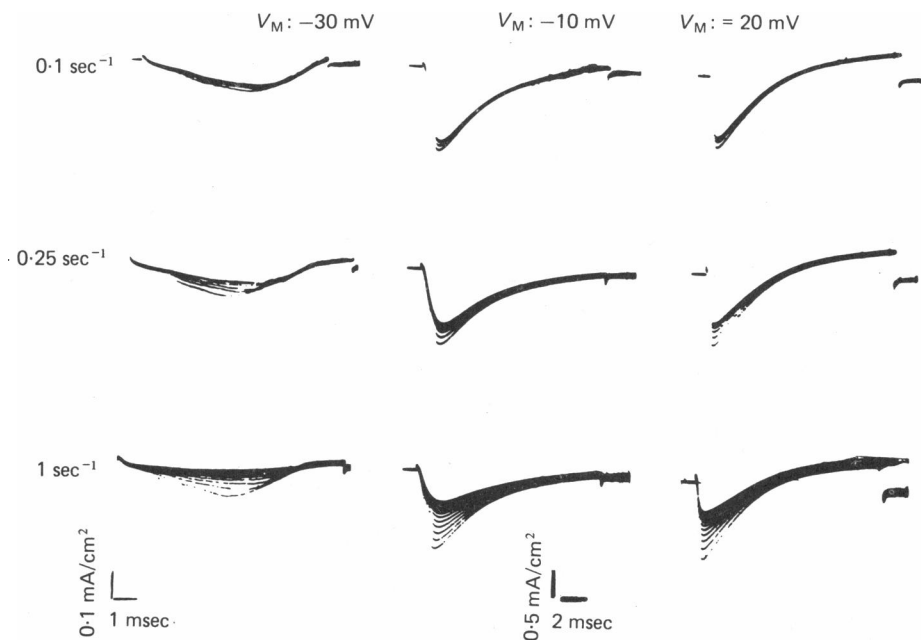


Fig. 5. Frequency response of the Na currents at various test potentials. Expt. 15-S-75. Axon bathed in K-free ASW.  $V_H = -80$  mV. Temp.  $5.5^\circ\text{C}$ . The axon was pulsed to the potential indicated above each column of records at the frequencies indicated.

repeated for pulses of various durations. In between the pulses the membrane was held at  $-80$  mV. The records demonstrate that the amount of current lost during repetitive pulsing increased as the length of the pulses was increased. However, the 22 msec pulse does not seem to produce much more decay than the one of 9 msec. This type of experiment was repeated for various pulse potentials in the range of  $-30$  to  $+90$  and for lengths up to 40 msec. In all cases the effect seems to reach saturation for pulses about 10–20 msec in duration at  $7\text{--}8^\circ\text{C}$ . In another experiment two pulses were applied to the membrane, separated by an interval of 200 msec. During the interval the membrane potentials were kept at  $V_H$ . This particular interval was chosen so that fast inactivation would be fully recovered. The membrane potential during the second pulse was  $+20$  mV. The membrane potential during the first pulse was either  $-20$ , 10 or 30 mV. The effect of the first pulse on the peak Na current during the second pulse was recorded for various durations of the first pulse. The results of this experiment demonstrated that for any of the three pre-pulse potentials, a 10, 20 or a 50 msec pre-pulse produced the same current for the second pulse: 80–85% of the current with no pre-pulse.

In order to simplify the quantitative analysis of the influence of membrane

potential on the phenomenon, two pulse experiments instead of multiple pulses were performed.

*Recovery of the Na conductance from inactivation by a depolarizing pulse*

Fig. 7 illustrates the recovery from inactivation induced by a single depolarizing pulse in *Myxicola* giant axons. The data for this curve was obtained from an

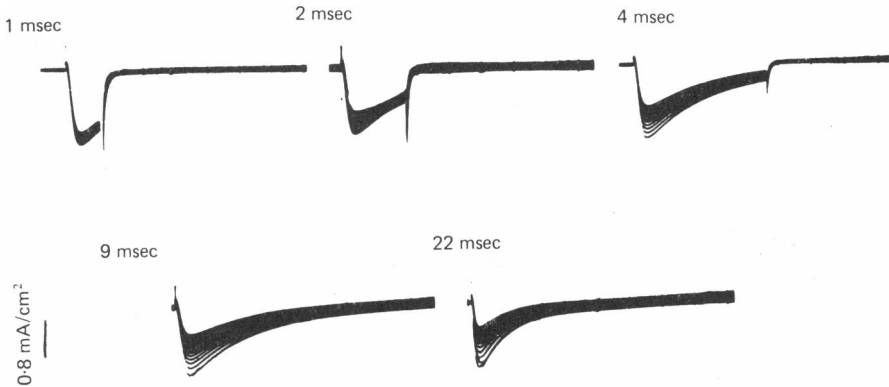


Fig. 6. Effect of pulse duration on the frequency response of the Na currents. Expt. 15-N-75. Axon bathed in K-free ASW + 10 mM-4-AP. Temp. 6 °C.  $V_H = -80$  mV. A depolarizing pulse to +20 mV was applied at a frequency of 1 sec<sup>-1</sup>. The duration of the pulse for each series is indicated.

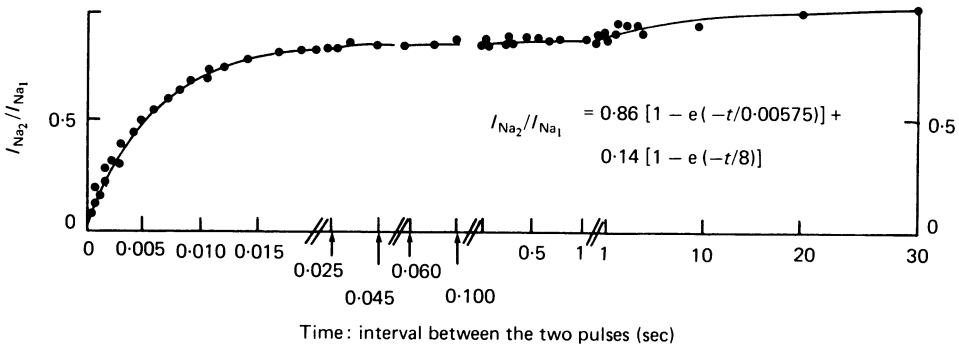


Fig. 7. Time course of recovery of Na current in *Myxicola* axons from inactivation by a single depolarizing pulse. Two equal pulses to +30 mV were separated by the time indicated on the abscissa. The ratio of the peak Na current for the second pulse over that for the first pulse is plotted on the ordinate. Axon bathed in K-free ASW.  $V_H = -70$  mV. Temp. 8 °C. Line fitted to the equation in the text.

experiment in which two identical depolarizing pulses were applied to the membrane. The holding potential for this particular experiment was  $-70$  mV. During the pulses the membrane potential was brought to +30 mV. The duration of each pulse was 19 msec. The interval between the pulses was varied as shown in the abscissa of Fig. 7. During the interval between pulses the membrane potential was equal to the holding potential. In the ordinate, is plotted the ratio of the peak Na current ( $I_{Na,2}$ )

during the second pulse over the peak sodium current during the first pulse ( $I_{Na,1}$ ). A 60 sec interval was allowed between each test.

In squid giant axons such an experiment produces a curve which is a single exponential (time constant =  $\tau_h$ ). In *Myxicola* the experimental points can be fitted by the equation:

$$\frac{I_{Na,2}}{I_{Na,1}} = a_1[1 - \exp(-t/\tau_1)] + a_2[1 - \exp(-t/\tau_2)], \quad (1)$$

where  $a_1 + a_2 = 1$ . For the experiment in Fig. 7:  $a_1 = 0.86$ ,  $a_2 = 0.14$ ,  $\tau_1 = \tau_h = 0.00575$  sec and  $\tau_2 = \tau_s = 8$  sec.

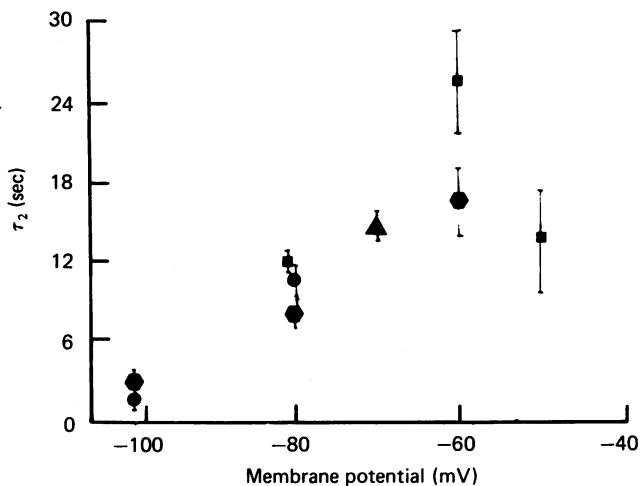


Fig. 8. Voltage dependence of the slow time constant of recovery of Na conductance from inactivation by a short pulse ( $\tau_2$ ). Values obtained from experiments such as that illustrated in Fig. 7 at various  $V_H$  values.

Similar experiments were performed for different holding potentials. In all cases the recovery curve was well fitted by the sum of two exponentials. The values of the fast time constant were similar to those reported by others for  $\tau_h$  (Goldman & Schaaf, 1973).

Fig. 8 illustrates the values for the slow time constant at different membrane potentials. The values of  $a_1$  and  $a_2$  in eqn. (1) appeared to be independent of the holding potential.

At holding potentials more positive than  $-50$  mV the currents become too small (due to  $h$  inactivation), making it difficult to estimate the slow time constant. Qualitative estimates suggest that it is still of the order of seconds at  $-40$  mV.

#### *Rates of onset of slow inactivation produced by a single depolarizing pulse*

In order to measure the rate of development of slow inactivation when produced by a short depolarizing pre-pulse, the following experimental scheme was followed. Two depolarizing pulses were applied to the membrane, separated by an interval of 250 msec. During this period the membrane was held at the holding potential (usually  $-80$  mV). This interval is approximately between ten and twenty times the time

constant for recovery of  $h$ , so it can be assumed that  $h$  has fully recovered. On the other hand, it is between  $\frac{1}{30}$  and  $\frac{1}{40}$  the time constant of recovery of the slow inactivation ( $s$ ), so very little recovery of  $s$  would have occurred. The length of the first pulse (pre-pulse) to a particular potential was increased. The ratio of the peak Na current during the second pulse (test pulse) for a pre-pulse of particular length over the value of the peak Na current for a test pulse in the absence of a pre-pulse

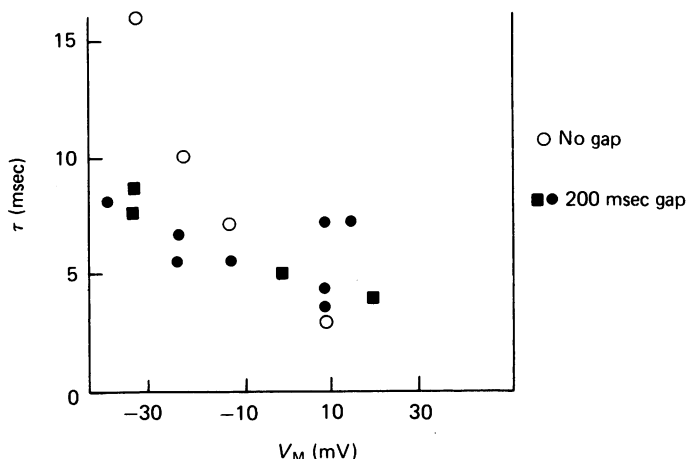


Fig. 9. Time constants of onset of fast and slow inactivation as function of membrane potential. Expt. 29-8-75 (squares) and 8-8-75 (circles). Temp. 7 °C. Two pulses to the potential indicated in the abscissa were applied from a  $V_H = -80$  mV. The duration of the first pulse was varied. The ratio of the peak Na current for a test pulse preceded by a pre-pulse over the peak Na current in the absence of a pre-pulse was plotted in a semilogarithmic paper as function of duration of the pre-pulse. The points were fitted to the best straight line, and from the slope the time constants were obtained. Filled symbols: a 200 msec interval at  $V_H$  was present between the two pulses. Open symbols: no interval between the two pulses.

was plotted against time. The curves thus obtained were fitted to a single exponential and the time constant estimated from the best fit. Individual tests were separated by one minute intervals at the holding potential.

The values of the time constant of onset obtained in this way for pre-pulses to various depolarizations are collected in Fig. 9.

#### *Effects of very long depolarizations*

The data illustrated above show that 15–20 % of the Na conductance enters a state of inactivation from which recovery is very slow when the membrane is depolarized for short periods of time.

In squid (Chandler & Meves, 1970; Rudy, 1978), in the node of Ranvier of frog sciatic nerve (Fox, 1976), and in *Myxicola* (Schauf, Pencek & Davis, 1976) depolarizations of the order of seconds result in a slow inactivation of the Na conductance from which recovery appears to be at similar rates to the slow inactivation described here.

Fig. 10 illustrates the effect of depolarizing pre-pulses when their length is extended to the order of seconds. The experiment is similar to those described earlier, i.e. a

two-pulse experiment with an interval of 250 msec between the two pulses. The curve illustrates that when the pre-pulse is extended to the time scale of seconds a further decrease in the Na current during the test pulse occurs which almost completely eliminates it.

The time constants of recovery from the later inactivation were compared to the values obtained for short depolarizations (Fig. 7) they are very similar at all potentials (see also Schaaf *et al.* 1976).

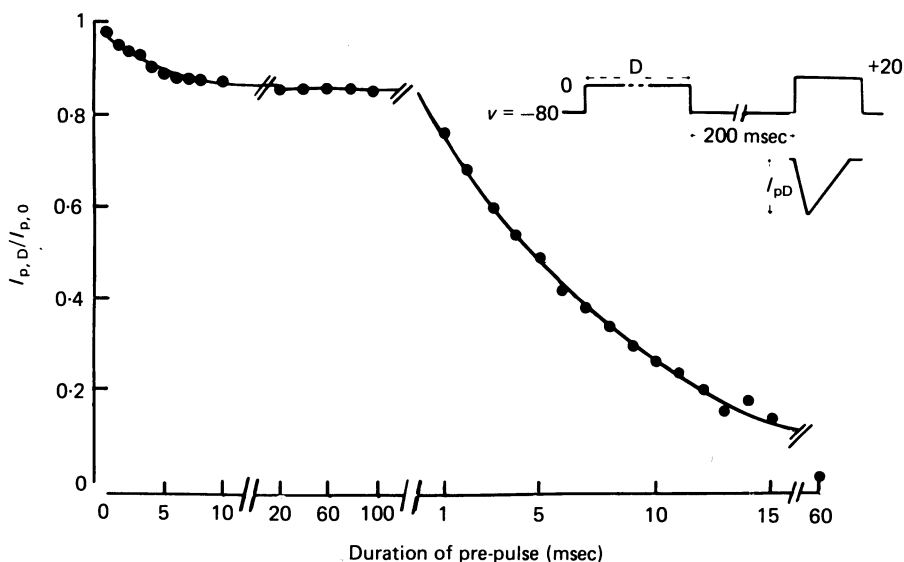


Fig. 10. Effect of a depolarizing test pulse to 0 mV on a test pulse to +20 mV applied 200 msec later. The duration of the pre-pulse was varied, and this is indicated on the abscissa. The ratio of the peak Na current during the test pulse over that in the absence of a pre-pulse is plotted on the ordinate.

These results suggest that the same *s* state is reached by 15–20% of the Na conductance during a short depolarization and by the rest if the depolarization is extended to several seconds. The kinetic model presented below attempts to explain these results.

#### DISCUSSION

##### *A kinetic model for slow inactivation*

The results presented above demonstrate that in *Myxicola* during a short depolarization (of physiological duration) the Na conductance enters an inactivated state from which recovery is very slow. The time constant of recovery is voltage dependent, changing about ten times in 30–40 mV. The onset of the inactivation is about 1000 times faster than the recovery and appears to be also voltage dependent (however, see below for coupling to open conductance). When brief and repetitive depolarizations are applied to the axons, the slowly recovering inactivation accumulates. A large percentage of the Na conductance can be blocked with a few depolarizations.

The model presented below does not attempt to provide a comprehensive kinetic

scheme for the Na channel conductance, but rather to propose the relationships that slow inactivation might have with other states in the channel to produce the observed changes.

Several results argue against the simplest type of models in which an independent first order process is incorporated to the other transitions in the channel. For example: (1) the time constants of onset and recovery are very far apart; recovery is still of the order of a second at  $-40$  mV while the onset is in the order of milliseconds at  $-30$  mV; it is difficult to conceive a steep enough voltage dependence which will change the rate constants for at least two orders of magnitude over an interval of only 10 mV, (2) for a first order voltage-dependent process the steady-state value of the process at a particular potential should be the same if approached by prolonging the duration of the reaction or by repetitive brief reaction times. However, as illustrated in Figs. 1 and 3 the process is accumulative and the effect observed when brief repetitive depolarizations are applied to the axon is much larger than that obtained by extending the duration of a single depolarization. For example, in Fig. 3 two brief pulses repeated at a frequency of  $1 \text{ sec}^{-1}$  produced a larger and faster decay than a longer single pulse repeated at the same frequency. For the case shown in Fig. 3B, there is no net ion movement for the second pulse, thus suggesting that it is the repetitive opening of the channel and not the ionic movement *per se* which leads to the accumulation of the inhibition. These results suggest that the onset of the phenomenon and its reversal use different pathways.

Only a small percentage of the Na conductance enters the slowly recovering state in a single brief depolarization. However, an inactivated state with similar rates of recovery is reached by most of the Na conductance if the depolarization is extended to several seconds (see Fig. 10 and Schauf *et al.* 1976). It is tempting to propose that the same state (*s*) is reached in the two conditions. The curve in Fig. 10 clearly shows two distinct steady states. I would like to propose that the inactivated state *s* can be reached by two distinct pathways with rates differing by two to three orders of magnitude. Clearly the amount that follows the fastest pathway is limited and as suggested by the model below it is proposed that this is due to competition with another reaction, the development of the fast inactivated state (*h* of Hodgkin & Huxley, 1952*b*).

Competition between the development of *h* and *s* was suggested by the similarities in rates between the developments of these two processes (see Fig. 9) and more clearly by the results shown in Fig. 11. The plot on the right compares the fraction of the Na current that has *not* undergone *h* inactivation with that that has *not* undergone *s* inactivation for pulses of increasing durations. The two scales are normalized to the maximum of current remaining for the longest pulse. The plot demonstrates that at any time the portion of channels undergoing *s* inactivation is proportional to the fraction that has undergone *h* inactivation. The experimental curves are shown on the left. This type of experiment allows for a better comparison between the time course of *h* and that of *s*, than the comparison of the time constants as done in Fig. 9, because the accumulative effects of *s* allow for a more accurate estimation of the fraction that enters *s*. The fraction in *s* after a single depolarization pulse is very small.

To summarize, I interpret the results as suggesting the presence in *Myxicola* axons of an inactivation phenomenon which is induced during a brief depolarization of

the membrane and recovers slowly after the membrane is hyperpolarized. If the membrane is repetitively depolarized, the inhibition accumulates. The amount capable of entering the slowly recovering inactivated state during a single depolarization is small and limited and further channels can be made available to undergo *s* inactivation if the channel is reopened. The results presented in Fig. 11 suggest that

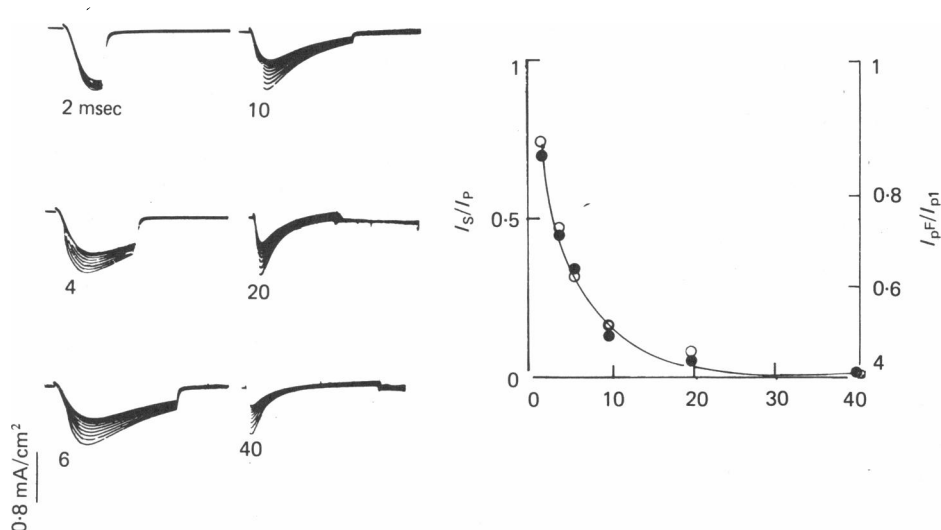
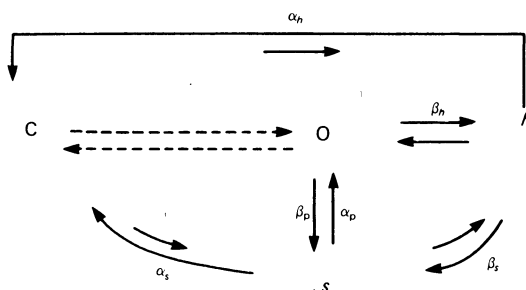


Fig. 11. Slow and fast inactivation increase proportionally as the length of a pulse is increased. *Myxicola* axon bathed in K-free ASW.  $V_H = -80$  mV. Temp.  $4.5^\circ\text{C}$ . Depolarizing pulses to  $-10$  mV were applied at 1/sec. The duration of the pulse is indicated next to each record. The plot on the right compares the variations of  $s_\infty$  (right ordinate, open symbols) and of  $h_\infty$  (left ordinate, filled symbols) as functions of the duration of the pulse.  $h_\infty$  is the value of the Na current at the end of the first pulse ( $I_s$ ) of each series over the peak Na current ( $I_p$ ) for the same pulse.  $s_\infty(I_{pF}/I_{p1})$  is the peak Na current at the end of each series ( $I_{pF}$ ) of pulses over that for the first pulse ( $I_{p1}$ ). Both curves are normalized to the minimum value of  $h$  or  $s$ .

it is *h* inactivation which prevents further channels from entering the *s* state, i.e. channels undergo either *h* or *s* inactivation during a short depolarization.

This model can be formally expressed as follows:



The kinetic scheme gives the relations of the slow inactivated state to other states in the channel (closed, open and rapidly inactivated) and does not include possible

intermediate steps. Armstrong & Bezanilla (1977) have proposed a kinetic scheme for the  $\text{Na}^+$  conductance that does not include slow inactivation and which has many more intermediate steps than the ones shown above. The forward rate constants dominate during depolarization and the backward rate constants during repolarization. Hypothetically dominating rate constants have been labelled.

The scheme stresses in particular the idea that slow inactivation can be reached both from open ( $O$ ) as well as from rapidly inactivated channels ( $h$ ). The same model can be applied both to *Myxicola* and squid giant axons (Rudy, 1978). In squid  $\beta_h$  is 100–1000 times larger than  $\beta_p$  and, therefore, open channels undergo preferentially fast inactivation. Slow inactivation is normally reached in this preparation through the  $h \rightarrow s$  reaction. After pronase treatment which blocks the  $O \rightarrow h$  reaction, slow inactivation remains but it develops three times faster (Rudy, 1978). Slow inactivation proceeds now through the  $O \rightarrow s$  reaction which is three times faster than the  $h \rightarrow s$  reaction. The same  $s$  state is reached before or after proteolytic treatment and, therefore, the recovery is identical. The recovery might show a complex time course because it might proceed through different pathways.

In *Myxicola* on the other hand  $\beta_p/\beta_p + \beta_h = 0.15$  and  $\beta_h$  is much larger than  $\beta_s$ . During each depolarizing cycle a significant fraction (15 %) of the channels enters the slow inactivated state from which recovery is very slow. Repetitive stimulation results in accumulation of channels in the  $s$  state. It is possible also to reach the same  $s$  state with a single depolarizing step if this is extended to several seconds; in this case inactivation proceeds through the slow  $h \rightarrow s$  reaction.

#### *Mechanisms of the frequency-dependent inhibition of the Na conductance*

Evidence has been presented that demonstrates that the frequency-dependent reversible decrease in Na conductance in *Myxicola* giant axons is not the result of ion accumulations or depletions due to increased ionic flux during depolarization. The phenomenon is voltage- rather than current-dependent. Furthermore, as shown in Fig. 3, the inhibition accumulates every time the channel is opened, independently of the presence or the direction of net ionic flux.

The gating currents in *Myxicola* axons show a frequency-dependent decrease in magnitude which corresponds to the decrease in ionic current described above and which is absent in gating or ionic currents in squid giant axons (Rudy, 1976a; Schauf, Bullock & Pencek, 1977; Bullock & Schauf, 1979). Gating currents are thought to represent the intramembranous movement of charges or dipoles in Na channels responsible for the opening and closing of the channels as the membrane potential changes (cf. Armstrong & Bezanilla, 1974; Keynes & Rojas, 1974). The frequency-dependent inhibition of the Na gating currents in *Myxicola* thus suggests that the slow inactivation described here is a process directly linked to the machinery that controls the Na channel gating.

The similarities between the data reported here and the phenomena observed when some local anaesthetics are applied to nerves are striking (Courtney, 1975; Hille, 1977). Further research should explore the possibility that the same molecular mechanism that operates in the case of drug action is responsible for the frequency-dependent effects observed in intact *Myxicola* axons. In particular, it will be of interest to test if the effects persist after the removal of the axoplasm.

*Significance of slow inactivation ( $h \rightarrow s$  reaction)*

Slow inactivation of the Na conductance is present in squid axons (Narahashi, 1964; Adelman & Palti, 1969; Chandler & Meves, 1970; Rudy, 1978), in the frog node of Ranvier (Fox, 1976; Peganov, Khodorov & Shishkova, 1973), in *Myxicola* giant axons (Schauf *et al.* 1976; Rudy, 1975, 1976*a*; and this paper) and in crayfish axons (Shrager, 1977). It also exists for the K conductance in squid and sciatic nerves (Lüttgau, 1960; Ehrenstein & Gilbert, 1966; Schwartz & Vogel, 1971), the Ca conductance of various tissues (Beeler & Reuter, 1970; Baker, Meves & Ridgway, 1973; Baker & Rink, 1975) and for a Cl conductance (Bennett, 1961), to mention only some examples. It thus seems that slow inactivation is a general property of voltage-dependent channels.

A question arises regarding the possible functional significance of slow inactivation since it is unlikely that an axon is depolarized for periods of time long enough for slow inactivation to take place to any appreciable degree. This may, however, not be true in densely packed regions of the central nervous system in which nervous activity leads to accumulation of  $K^+$  ions in the extracellular space which in turn could maintain the membrane depolarized for long periods of time (Nicholson, 1979). The time course of  $K^+$  wash-out from the brain as measured with ion-selective electrodes is in the proper range for slow inactivation to take place (Nicholson, 1979).

*Significance of the 'open-slow inactivated' shunt pathway*

As proposed in another paper (Rudy, 1978), the kinetic model presented above might not be unique to *Myxicola* giant axons. In squid giant axons (Rudy, 1976, 1978) the shunt pathway open-slow inactivated is too slow and thus does not produce any apparent frequency-dependent blockage of the Na conductance. I would like to hypothesize that the kinetic model presented earlier, in particular the idea that slow inactivation can be reached either from the open or from the  $h$  inactivated states is also general to Na channels. If this turns out to be the case, slow inactivation could play a fundamental role in determining functional variations in Na-channel-containing membranes. Thus the ratio of the rate constants  $\beta_h$  and  $\beta_p$  will determine the frequency response of a particular excitable membrane at frequencies far beyond those in which  $h$  and K conductance normally play a role and *without altering the shape of individual action potentials*. The rate of the pathway  $h$  inactivated to slow inactivated will determine the length of time that a particular membrane can tolerate being under continuous depolarization without undergoing significant slow inactivation. The rate of recovery from slow inactivation ( $\alpha_s$  in the model) will determine the time scale in which adaptation resulting from frequent stimulation or long term depolarization will affect excitability.

In particular, variations in the rate of the open-slow inactivated shunt pathway, which so distinctly alters the behaviour of *Myxicola* giant axons, should lead to interesting possibilities. Nerves with fast shunt pathways such as in *Myxicola* could serve as frequency filters or frequency transducers.

Furthermore, the simultaneous and competitive presence of  $h$  and  $s$  (where recovery from  $s$  is slow compared to its onset as in *Myxicola*) results in an observable accumulative adaptation, so that the system has a 'memory'. Such a system could

serve to 'count' impulses where the initial number in the count is determined by the immediate previous history of the axon. In contrast,  $h$  inactivation alone will only give accumulative adaptation at much higher frequencies and only if the membrane is maintained somewhat depolarized in between spikes so that no significant recovery of  $h$  takes place, and it may alter the shape of individual action potentials. In eccentric cells in *Limulus* eye, Fuortes & Mantegazzini (1962) have described a frequency-dependent adaptation which could well be the result of a fast shunt pathway to slow inactivation as described here for *Myxicola*. In frog muscle Lüttgau (1965) has described a fatigue of the action potential with a time course expected for the process described here. Interestingly, the fatigue in muscle was dependent on the metabolic state of the cell. As mentioned in the Introduction, adaptation is common in Annelids and it is very possible that other animals of this class have a similar adaptation to that in *Myxicola*. Evidence exists that suggests this might be the case in *Nereis* (Horridge, 1959).

#### *Functional role of the frequency-dependent adaptation in Myxicola*

I want to advance the following hypothesis for what the role of the frequency-dependent habituation in the behaviour of *Myxicola* and other Annelids might be.

In Annelids the synaptic junction with the muscles that contract under stimulation of the giant nerves habituates rapidly. In contrast to the adaptation of the axon this occurs at much lower frequencies, and in a certain range of frequencies it is the number of stimulations rather than the frequency that determines the degree of habituation. Also compared to the axon the recovery of the synapse is very much slower (Dales, 1970). It has been suggested that the role of this habituation is to maintain a balance between the amount of time that the animal spends buried and its exit in search of food and oxygen.

The contraction produced by the stimulation of the giant fibres is quite strong and it is conceivable that this in turn would re-stimulate the giant axon through spread from quickly stretching lateral branches. A continuous stimulation of the giant axon could result in an undesirable inhibition of the synaptic junction with the muscles. Therefore, the axon requires a system that would lead to absence of response if it is stimulated at a high enough frequency.

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#### REFERENCES

- ADELMAN, JR., W. J. & PALT, Y. (1969). The effects of external potassium and long duration voltage conditioning on the amplitude of sodium currents in the giant axon of the squid, *Loligo pealei*. *J. gen. Physiol.* **54**, 589–606.
- ARMSTRONG, C. M. & BEZANILLA, F. (1974). Charge movement associated with the opening and closing of the activation gates of the Na channels. *J. gen. Physiol.* **63**, 533–552.
- ARMSTRONG, C. M. & BEZANILLA, F. (1977). Inactivation of the sodium channel. II. Gating current experiments. *J. gen. Physiol.* **70**, 567–590.

- BAKER, P. F., MEVES, H. & RIDGWAY, E. B. (1973). Calcium entry in response to maintained depolarization of squid axons. *J. Physiol.* **231**, 527-548.
- BAKER, P. F. & RINK, T. J. (1975). Catecholamine release from bovine adrenal medulla in response to maintained depolarization. *J. Physiol.* **253**, 593-620.
- BEELER, G. W. & REUTER, H. (1970). Membrane calcium current in ventricular myocardial fibres. *J. Physiol.* **207**, 191-209.
- BEGENISICH, T. (1975). Magnitude and location of surface charges on *Myxicola* giant axons. *J. gen. Physiol.* **66**, 47-65.
- BENNETT, M. V. L. (1961). Modes of operation of electric organs. *Ann. N. Y. Acad. Sci.* **94**, 458-509.
- BINSTOCK, L. & GOLDMAN, L. (1969). Current and voltage clamp studies on *Myxicola* giant axons. *J. gen. Physiol.* **54**, 730-740.
- BULLOCK, J. O. & SCHAUF, C. L. (1979). Immobilization of intramembrane charge in *Myxicola* giant axons. *J. Physiol.* **286**, 157-171.
- CHANDLER, W. K. & MEVES, H. (1970). Slow changes in membrane permeability and long lasting action potentials in axons perfused with fluoride solutions. *J. Physiol.* **211**, 707-728.
- COURTNEY, K. R. (1975). Mechanism of frequency-dependent inhibition of sodium currents in frog myelinated nerve by the lidocaine derivative. GEA 968, *J. Pharmac. exp. Ther.* **195**, 225-236.
- DALES, P. R. (1970). *Annelids*. London: Hutchinson.
- EHRENSTEIN, G. & GILBERT, D. L. (1966). Slow changes in potassium permeability in squid giant axon. *Biophys. J.* **6**, 533.
- FOX, J. M. (1976). Ultra-slow inactivation of the ionic currents through the membrane of myelinated nerve. *Biochim. biophys. Acta* **426**, 232-244.
- FRANKENHAEUSER, B. & HODGKIN, A. (1956). The after effects of impulses in the giant nerve fibres of *Loligo*. *J. Physiol.* **131**, 341-376.
- FUORTES, M. G. & MANTEGAZZINI, F. (1962). Interpretation of the repetitive firing of nerve cells. *J. gen. Physiol.* **45**, 1163-1179.
- GOLDMAN, L. & SCHAUF, C. L. (1973). Quantitative description of sodium and potassium currents and computed action potentials in *Myxicola* giant axons. *J. gen. Physiol.* **61**, 361-384.
- HILLE, B. (1977). Local anesthetics. Hydrophilic and hydrophobic pathways for the drug receptor reaction. *J. gen. Physiol.* **69**, 497-515.
- HODGKIN, A. L. & HUXLEY, A. F. (1952a). The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *J. Physiol.* **116**, 497-506.
- HODGKIN, A. L. & HUXLEY, A. F. (1952b). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117**, 500-554.
- HORRIDGE, G. A. (1959). Analysis of the rapid responses of *Nereis* and *Harmothae*. *Proc. R. Soc. B* **150**, 245-262.
- KEYNES, R. D. & ROJAS, E. (1974). Kinetics and steady-state properties of the charged system controlling sodium conductance in the squid giant axon. *J. Physiol.* **239**, 393-434.
- LÜTTGAU, H. C. (1960). Das Kalium-Transport system am Ranvier Knoten isolierter markhaltiger Nervenfasern. *Pflügers Arch. ges. Physiol.* **271**, 613-633.
- LÜTTGAU, H. C. (1965). The effect of metabolic inhibitors on the fatigue of the action potential in single muscle fibres. *J. Physiol.* **178**, 45-67.
- NARAHASHI, T. (1964). Restoration of action potential by anodal polarization in lobster giant axons. *J. cell. comp. Physiol.* **64**, 73-96.
- NICHOLSON, C. (1979). Brain-cell microenvironment as a communication channel. In *The Neurosciences. Fourth Study Program*, ed. SCHMITT, F. O. & WORDEN, F. G., pp. 457-476. Massachusetts: MIT Press.
- PEGANOV, E. M., KHODOROV, B. I. & SHISHKOVA, L. D. (1973). Slow sodium inactivation in the Ranvier node membrane; role of external potassium. (In Russian.) *Bull. exp. Biol. Med.* **76**, 15-19.
- PELHATE, M. & PICHON, Y. (1974). Selective inhibition of potassium current in the giant axon of the cockroach. *J. Physiol.* **242**, 90P.
- RUDY, B. (1975). Slow recovery of the inactivation of sodium conductance in *Myxicola* giant axons. *J. Physiol.* **249**, 22-24P.
- RUDY, B. (1976a). Sodium gating currents in *Myxicola* giant axons. *Proc. R. Soc. B* **193**, 469-475.
- RUDY, B. (1976b). Studies on Inactivation Mechanisms in Nerves. Ph.D. Thesis. University of Cambridge.
- RUDY, B. (1977). A kinetic model for slow inactivation in nerves. *Biophys. J.* **17**, 45a.

- RUDY, B. (1978). Slow inactivation of sodium conductance in squid giant axons. Pronase resistance. *J. Physiol.* **283**, 1-21.
- SCHAUF, C. L., BULLOCK, J. O. & PENCEK, T. L. (1977). Characteristics of sodium tail currents in *Myxicola* axons. Comparison with membrane asymmetry currents. *Biophys. J.* **19**, 7-28.
- SCHAUF, C. L., PENCEK, T. L. & DAVIS, F. A. (1976). Slow sodium inactivation in *Myxicola* axons. *Biophys. J.* **16**, 771-778.
- SCHWARZ, J. R. & VOGEL, W. (1971). Potassium inactivation in single myelinated nerve fibres of *Xenopus laevis*. *Pflügers Arch.* **330**, 61-73.
- SHRAGER, P. (1977). Slow sodium inactivation in nerve after exposure to sulfhydryl blocking reagents. *J. gen. Physiol.* **69**, 183-202.